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13. ABSTRACT ***(Maximum 200 Words)***

A central issue in cancer biology is how a tissue maintains appropriate cell numbers. Apoptosis plays an essential role in controlling cell numbers by inducing the deaths of extra cells. The Bcl-2 family proteins are conserved apoptosis regulators. Overexpression of *bcl-2* can cause B-cell lymphoma and likely other human cancers, including breast cancer. How Bcl-2 family proteins act to regulate apoptosis is poorly understood.

The major goal of this project is to carry out *in vitro* selection (SELEX) to identify high affinity and specificity small RNA ligands (aptamers) for CED-9, a *C. elegans* Bcl-2-like apoptosis inhibitor, and three important mammalian Bcl-2 family proteins, and then use isolated apatmers to study how CED-9/Bcl-2 family proteins regulate apoptosis.

We have developed and improved the SELEX method and have made progresses and obtained promising results on isolating aptamers for CED-9 and mammalian Bcl-2 proteins. We will continue the screens until high affinity aptamers for CED-9 and other Bcl-2 family proteins are isolated.

The studies described here will provide a novel approach and generate new reagents for studying the mechanisms of Bcl-2 family proteins in apoptosis. These studies may also yield potential diagnostic reagents and may generate potent apoptosis-inducing compounds useful in the detection and treatment of breast cancer.

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Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....6

Reportable Outcomes.....6

Conclusions.....6

References.....7

Appendices.....8

Introduction

A central issue in our understanding of cancer biology is how a tissue or organ maintains the appropriate number of cells. As a normal aspect of animal development and homeostasis, programmed cell death (apoptosis) plays an essential role in maintaining the physiological balance of appropriate cell numbers by opposing uncontrolled cell proliferation. Programmed cell death is controlled and executed by a cell death pathway that is highly conserved from the nematode *C. elegans* to humans. At the heart of this pathway is a family of conserved cell death regulators, first defined by the human anti-apoptotic proto-oncogene *bcl-2*. The Bcl-2 family contains both anti-apoptotic and pro-apoptotic members that may regulate the appropriate activation of apoptosis by interacting with and modulating the activities of other cell death regulators or by affecting the membrane permeability of important organelles such as mitochondria. Abnormal inactivation of apoptosis, such as overexpression of *bcl-2*, can lead to uncontrolled cell growth and contribute to the pathogenesis and progression of various human cancers including breast cancer and tumor resistance to chemo- or radio- therapies. Thus, elucidation of the molecular mechanisms by which Bcl-2 family proteins regulate apoptosis is critical for improving our knowledge of cancer biology. Furthermore, identification of small and potent molecular ligands for Bcl-2 family proteins that can be used to activate or inactivate apoptosis at our will can greatly facilitate the development of new therapeutic methods in the treatment and prevention of breast cancer.

Our objective in this study is to carry out *in vitro* selection (SELEX) to identify high affinity and specificity small RNA ligands (aptamers) for the *C. elegans* cell death inhibitor CED-9 (an invertebrate prototype of Bcl-2 proteins) and three important mammalian Bcl-2 family proteins. We will then use *C. elegans*as a key experimental system and the isolated apatmers to study how CED-9/Bcl-2 family proteins regulate apoptosis and to screen for potent apatmers that potentially can be applied diagnostically or therapeutically in the detection, prevention, or treatment of human cancer.

Three major goals of this study are: 1) Development of the SELEX method and isolation of apatmers for the *C. elegans* cell death inhibitor CED-9; 2) Characterization of CED-9 apatmers and their effects on *C. elegans* cell death; 3) Isolation and characterization of apatmers for mammalian Bcl-2 family proteins and their effects on mammalian cell death.

We have developed an effective SELEX method to isolate apatmers for CED-9/Bcl-2 proteins. We will use aptamers isolated to probe the functional domains of CED-9/Bcl-2 proteins, the interactions of CED-9/Bcl-2 proteins with other cell death regulators, and the mechanistic basis by which CED-9/Bcl-2 family regulate cell death. We will screen for apatmers that can potently increase or decrease the activity of CED-9/Bcl-2 proteins *in vivo* and characterize the mechanistic and structural basis of such interference by biochemical and structural biological analyses.

The studies described here will provide a novel approach and generate many new useful reagents for studying the mechanisms of Bcl-2 family proteins in apoptosis, which thus far remain poorly understood. But more importantly, these studies may yield simple and powerful diagnostic reagents for the detection of breast cancer and may generate potent apoptosis-inducing compounds and provide important structural insights for designing new therapeutic drugs in treatment of breast cancer.

Body:

Task 1. **Development of the SELEX method and isolation of apatmers for the *C. elegans* cell death inhibitor CED-9**

a. Develop an effective and successful SELEX protocol

The graduate student, Jay Parrish, who initiated this project, was close to finish his Ph.D. study in my laboratory when this grant was awarded. A new postdoctoral fellow, Dr. Chonglin Yang, who has a strong background on RNA work, took over the project when he joined my laboratory last December. Dr. Yang started the project by developing several strategies to improve the SELEX method originally designed by Jay Parrish, who had tried to use this method to isolate aptamers for the SXL protein (sex lethal protein), a *Drosophila* splicing factor. Since the SELEX approach is a very sensitive screen, it demands high purity of the target protein to be used in the screen in order to obtain high specificity aptamers. Dr. Yang has spent quite a long time trying to obtain very pure CED-9 protein preparations, using several steps of chromatography purification. He eventually was able to obtain more than 99% pure of two different CED-9 proteins, His_6CED-9(1-251) and His_6CED-9(68-251) (Figure 1). The second strategy that he used to improve the specificity of his SELEX screen is to conduct the SELEX screens in parallel using these two different CED-9 proteins, both of which have been shown to be sufficient to bind *C. elegans* cell death activator CED-4 as well as the cell death activator EGL-1 [1, 2], two key regulators of *C. elegans* apoptosis [3, 4]. After he isolates aptamers for both CED-9 proteins, he can then compare the products that he isolates to see which ones are specific to the CED-9 protein.

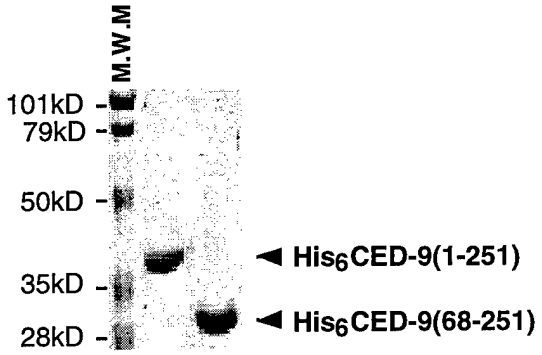

Figure 1. Purified CED-9 proteins. M.W.M. stands for moleuclar weight marker. 3 μg of proteins each are resolved on 10% SDS PAGE and stained with coomassie blue.

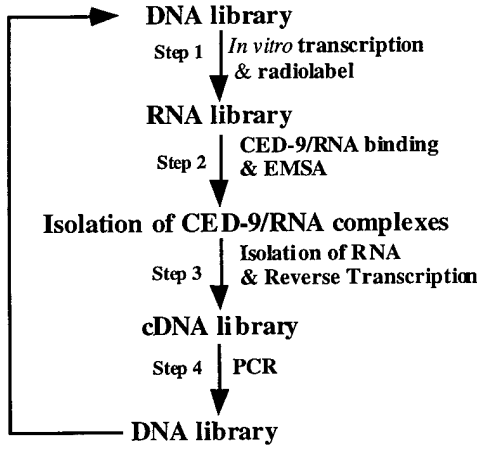

Figure 2 Scheme of SELEX. RNA aptamers are selected on the basis of their ability to bind His_6CED-9(68-251). His_6CED-9(68-251) concentration used is progressively decreased to select high-affinity aptamers.

The SELEX scheme that Dr. Yang used is shown in Figure 2. The oligonucleotide library used contains a central region of 50 randomized nucleotides flanked at both ends by constant sequences. For the first round of SELEX approximately 10^{15} unique sequences were represented. Each round of SELEX consists of the following steps: radio-labeled RNAs were synthesized by *in vitro* transcription reaction in the presence of ^{32}P-ATP and the T7 RNA polymerase using the oligonucleotide library as templates (step 1) and then incubated with CED-9 before they were applied to native polyacrylamide gels (PAGE) (Step 2). CED-9/RNA complexes were isolated from the EMSAs (electrophoretic mobility shift assay) and the bound RNAs were eluted from the gel slides and reverse transcribed to generate cDNAs (step 3), which were then PCR-amplified to generate a new oligonucleotide library enriched in DNAs encoding RNAs

with higher binding affinities for CED-9 (step 4, Figure 2). Normally, 12-14 rounds of SELEX need to be done to yield aptamers that bind a target protein with high affinity (1-10 nM Kds).

b. Isolate apatmers for CED-9

So far, we have done six rounds of SELEX on both His_6CED-9(1-251) and His_6CED-9(68-251) proteins, and as expected, the Kds of the libraries for both CED-9 proteins are steadily increasing (the Kd of the starting library for CED-9 is in the range of 10 mM). We will do another 6-8 rounds of SELEX on these two CED-9 proteins.

c. Sequence the apatmers isolated

d. Compare and analyze the sequences of apatmers and categorize the apatmers sequenced.

So far we have not yet advanced to these two steps but expect to get to them soon.

Task 2. **Characterization of CED-9 apatmers and their effects on *C. elegans* cell death.**

We have not yet advanced to this task but expect to get to it soon.

Task 3. **Isolation and characterization of apatmers for mammalian Bcl-2 family proteins and their effects on mammalian cell death.**

a. Isolate apatmers for Bcl-2, Bcl-xL, and Bax

So far, we have constructed two different kinds of bacterial expression vectors for these three mammalian Bcl-2 family proteins, all of which have been tagged with six consecutive Histidines or GST (glutathione S transferase) for affinity chromatography purification. We are now in the process of purifying these proteins using several chromatography steps. Once we have these three proteins purified to homogeneity, we will proceed to conduct the SELEX screens on these proteins to isolate aptamers specific for each protein.

Key Research Accomplishments

- We have successfully purified high quality of CED-9 proteins for the SELEX screens
- We have developed and improved the SELEX method to isolate aptamers for the CED-9 proteins
- We have conducted six rounds of the SELEX screens for two CED-9 proteins and got promising results.
- We have constructed bacterial expression vectors to express and purify three mammalian Bcl-2 family proteins.

Reportable Outcomes

Dr. Chonglin Yang, who originally was a researcher in the field of RNA regulation, is able to switch field to study apoptosis in *C. elegans*, a new model organism and a new research field for him. He will benefit greatly from such a switch in his training to become an independent scientist.

Conclusions

We have developed and improved the SELEX method to isolate high affinity and specificity small RNA ligands (aptamers) for a target protein. We have made progresses and obtained promising results on our efforts to isolate aptamers for the *C. elegans* cell death inhibitor CED-9 and its mammalian cell death homologues. Once these aptamers are isolated, we will use these aptamers to probe the

interactions of CED-9/Bcl-2 with other apoptotic regulators and the functioning mechanisms of CED-9/BCl-2 family proteins in regulating apoptosis. We will explore the possibilities that some of these aptamers can be used to perturb (induce or block) apoptosis in *C. elegans* and in human cells and thus can be used or modified to become potential diagnostic or even therapeutic agents for the detection or treatment of cancers, which most often are caused by inappropriate apoptosis.

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Appendices

Curriculum Vitae of a key personnel (Dr. Chonglin Yang)

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Chonglin Yang	Postdoctoral Associate

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Sichuan Agricultural University (Sichuan, China)	B.S.	1988	Agronomy
Kunming Institute of Botany (Kunming, China)	M.S.	1995	Plant genetics
Peking University (Beijing, China)	Ph.D.	1998	Molecular biology

A. Positions. List in chronological order previous positions, concluding with your present position.

1991-1995	**M.S. Candidate**, Kunming Institute of Botany, Chinese Academy of Sciences Kunming, China. Laboratory of Jianmin Zhang. Induction and selection of auxotrophic mutants from the protoplasts of *Lentinus edodes.*
1995-1998	**Ph.D. Candidate**, Peking University, Beijing, China. Laboratory of Dr. Zhangliang Chen Molecular cloning and characterization of novel disease-resistance genes in rice.
1998-1999	**Postdoctoral Fellow.** International School for Advanced Studies, Trieste, Italy. Laboratory of Dr. Andrew Bradbury. Characterization, expression and purification of GABA delta subunit in rat neonatal hippocampus.
1999-2001	**Postdoctoral Fellow.** University of Maryland, Baltimore, Maryland. Laboratory of Dr. France Carrier. Functional study of RNA-binding proteins in cellular responses to genotoxic stress.
2001-present	**Postdoctoral Fellow.** University of Colorado, Boulder, Colorado. Laboratory of Dr.Ding Xue. Selection of aptamers for CED-9/Bcl-2 family cell death regulators and their application in study of apoptosis regulation and drug design for breast cancer.

B. Honors. List any honors. Include present membership on any Federal Government public advisory committee.

1992	Third prize for excellent scientific article, 1992, Kunming Institute of Botany, the Chinese Academy of Sciences.
1996	"Guanghua Scholarship" for excellent Ph.D. student, Peking University
1998	Excellent Ph.D. dissertation, Peking University

C. Selected peer-reviewed publications (in chronological order). Do not include publications submitted or in preparation.

1. Chonglin Yang, Dony A Maiguel and France Carrier (2002). Identification of nucleolin and nucleophosmin as genotoxic stress-responsive RNA binding proteins. **Nucleic Acids Res.** 30:2251-2260.

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